

Leptospira reservoirs among wildlife in Brazil: Beyond rodents

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ABSTRACT

Leptospirosis is a disease of great importance in tropical regions. Infection occurs mainly through contact with water contaminated with the urine of infected animals, especially that of rodents. Despite the diversity and abundance of wild fauna in Brazil, little is known about the role of other wild species in the epidemiology of leptospirosis. This study aimed to investigate new reservoirs of *Leptospira* among wildlife in Brazil, using serological and molecular diagnoses in a large-sized sample. Biological samples were collected from 309 free-ranging mammals, belonging to 16 species. The majority of the animals included were opossums (*Didelphis albiventris*) and coatis (*Nasua nasua*). Blood and urine samples were subjected to the microscopic agglutination test (MAT) and real-time PCR, respectively. Genetic characterization of genomospecies was performed using PCR amplicons. Statistical analysis was applied to test associations between positive diagnoses and age, sex, season and type of environment. The prevalence of infection found via MAT and PCR was 11% and 5.5%, respectively. If these tests are taken to be complementary, the overall prevalence was 16%. The most common serogroups were Djasiman and Australis, while *L. santarosai* was the prevalent genomospecies. Significant differences in prevalence between animal species were observed. Greater risk of infection was detected among adult opossums than among young ones. The influence of each serogroup and genomospecies was tested for the same variables, and this revealed higher risk of infection by *L. santarosai* among male opossums than among females. The present study highlights the exposure and carrier status of several wild species in Brazil and it indicates that coatis and other carnivores are priorities for further investigations.

1. Introduction

Leptospira infection is common worldwide. Virtually all mammal species are susceptible and able to maintain the microorganism in their tissues, and there is evidence that even reptiles and birds can harbor leptospires (Biscola et al., 2011; Jobbins and Alexander, 2015). Infection occurs through direct contact with secretions of an infected animal, especially urine, or indirectly through contact with contaminated water. The infection is usually asymptomatic or only results in mild and nonspecific symptoms. However, in some cases leptospirosis may present as a severe illness in both animals and humans, leading to death (Levett, 2001).

Control over leptospirosis is based on interruption of direct and indirect transmission of infection. Specific methods can be used according to the animal population, environmental conditions, infecting serovars, costs, access to animals and species involved, among many other factors (Ellis, 2015). Regardless of the method to be used, one crucial step towards controlling leptospirosis is to identify the animal hosts, since they are responsible for shedding the bacteria and contaminating the environment (Haake and Levett, 2015).

Many studies have identified wild species as reservoirs of leptospirosis, including rodents, bats, foxes, sea lions and capybaras (Prager et al., 2013; Scialfa et al., 2013; Dietrich et al., 2015; Albuquerque et al., 2017). The Norway rat (*Rattus norvegicus*) is considered to be the

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Table 1
Absolute and relative frequencies of *Leptospira* infection in free-ranging wild species from the municipality of Botucatu, Brazil.

Species	Number tested	MAT positive	CI	PCR positive	CI	MAT + PCR positive	CI
Capuchin monkey (<i>Sapajus nigritus</i>)	2	0 (0%)	–	2 (100%)	–	2 (100%)	–
Coati (<i>Nasua nasua</i>)	56	15 (26.8%)	15–38%	5 (8.9%)	1–16%	20 (35.7%)	23–48%
Crab-eating fox (<i>Cerdocyon thous</i>)	4	3 (75%)	–	0 (0%)	–	3 (75%)	–
Crab-eating raccoon (<i>Procyon cancrivorus</i>)	1	1 (100%)	–	0 (0%)	–	1 (100%)	–
European hare (<i>Lepus europaeus</i>)	2	0 (0%)	–	0 (0%)	–	0 (0%)	–
Giant anteater (<i>Myrmecophaga tridactyla</i>)	6	0 (0%)	–	0 (0%)	–	0 (0%)	–
Gray brocket (<i>Mazama gouazoubira</i>)	5	0 (0%)	–	0 (0%)	–	0 (0%)	–
Hoary fox (<i>Lycalopex vetulus</i>)	4	2 (50%)	–	0 (0%)	–	2 (50%)	–
Lesser grison (<i>Galictis cuja</i>)	6	4 (67%)	–	0 (0%)	–	4 (67%)	–
Maned wolf (<i>Chrysocyon brachyurus</i>)	3	2 (67%)	–	0 (0%)	–	2 (67%)	–
Nine-banded armadillo (<i>Dasypus novemcinctus</i>)	4	1 (25%)	–	1 (25%)	–	1 (25%)	–
Opossum (<i>Didelphis albiventris</i>)	195	3 (2%)	0–4%	7 (3.5%)	0–6%	10 (5%)	2–8%
Porcupine (<i>Sphiggurus villosus</i>)	13	0 (0%)	0–25%	2 (15.3%)	1–45%	2 (15.3%)	1–45%
Puma (<i>Puma concolor</i>)	2	0 (0%)	–	0 (0%)	–	0 (0%)	–
Southern tamandua (<i>Tamandua tetradactyla</i>)	5	1 (20%)	–	0 (0%)	–	1 (20%)	–
Tayra (<i>Eira Barbara</i>)	1	1 (100%)	–	0 (0%)	–	1 (100%)	–
Total	309	33 (11%)	7–14%	17 (5.5%)	2–8%	49 (16%)	12–20%

MAT: microscopic agglutination test.

PCR: real time polymerase chain reaction.

CI: confidence interval.

CI was not calculated for species with few animals sampled.

most important host of human leptospirosis (Levett, 2001). However, there is a lack of data regarding the importance of other wild species for public health. Recent studies have pointed towards wild boars, monkeys and tenrecs as possible transmitters of leptospirosis to humans (Jansen et al., 2006; Dreyfus et al., 2016; Lagadec et al., 2016). Studies on this topic in Brazil are very scarce, and so far have also pointed towards rodents as the main wild reservoirs (Faria et al., 2008; Silva et al., 2010; Costa et al., 2014).

Despite the diversity and abundance of fauna in Brazil, little is known about the role of wild species (other than rodents) in the epidemiology of leptospirosis. The literature is sparse and limited to studies that either only involved small numbers of animals, or only involved few species, or only used serological methods (Fornazari and Langoni, 2014). To overcome these limitations, we conducted a cross-sectional study using a large-sized sample, several animal species and molecular diagnostic techniques. Our main goal was to identify new reservoirs of *Leptospira* among wildlife in Brazil.

2. Materials and methods

2.1. Study design

This study was conducted in the municipality of Botucatu (22°53'09" S 48°26'42" W), which is located in the center the state of São Paulo, the most populated and economically developed region of Brazil. Previous studies have confirmed that *Leptospira* infection occurs widely in several domestic species in this region (Langoni et al., 2008; Coiro et al., 2012; Fornazari et al., 2012; Kikuti et al., 2012). Botucatu has a population of approximately 140,000 habitants, annual rainfall of 1358 mm, annual mean temperature of 20.7 °C, intensive land use for eucalyptus plantations, agriculture and livestock, massive fragmentation of native forest and atypical strong winds (CEPAGRI; IBGE).

The sample size was calculated using the OpenEpi software (www.openepi.com), with 95% confidence interval and considering the prevalence to be 12.6% as reported in the study published by Souza Junior et al. (2006). The minimum sample was thus calculated as 163 animals. However, because of major differences between Souza Junior's study and ours, such as animal species, environmental features and laboratory diagnosis, the appropriateness of their reported prevalence for our study was uncertain. We did not find any references in the literature that were more suitable. Therefore, to be safe, we increased the numbers of animals sampled to 300.

Only free-ranging mammals were included in the study. They were sampled between April 2012 and January 2014 using two methods: (1) capture in their natural habitat and (2) through following the daily routine of the Center for Medicine and Wildlife Research (CEMPAS), São Paulo State University (UNESP), Botucatu. All the procedures had received approval from the Chico Mendes Institute for Biodiversity Conservation (ICMBio, license n° 33162-2) and the Ethics Committee of UNESP (protocol n° 10/2012).

2.2. Animals and samples

Animals were caught in two rural areas located close to the urban perimeter of Botucatu: the Botanical Garden (22°53'08" S 48°29'58" W) and two farms with continuous landholdings, named Edgardia and Lageado (22°49'56" S 48°25'38" W). The environments of the two areas were similar, characterized by several small patches of Atlantic semi-deciduous forest, surrounded mainly by cattle ranching and few sugar cane plantations. Streams and small rivers were frequent on both areas, including inside the forest patches and on the grazing areas. Tomahawk traps were assembled inside the forest patches in the mornings, baited with fruits and checked 24 h later.

The animals were anesthetized in the field using ketamine and midazolam at different doses according to the species. Blood and urine samples were collected by means of venipuncture and cystocentesis/urethral catheterization, respectively. When needed, an intravenous injection of furosemide, together with fluid replacement, was used to increase urine production. The animals were identified individually using metal ear tags to avoid repeated sampling. They were then placed inside the traps again and were only released after complete recovery from the effects of the anesthesia. The biological samples were transported to the Department of Veterinary Hygiene and Public Health (DHVSP) of UNESP. Animals sent to CEMPAS were sampled during ambulatory procedures.

Age, sex, type of environment and sampling date were recorded for each animal. Age was classified as young or adult according to animal size and tooth eruption. Because some animals sent to CEMPAS were rescued inside the city, the type of environment was classified as "rural" or "urban". To correlate infection with season, the sampling date of each animal was adjusted, since laboratory diagnostic methods cannot yield positive results regarding infection on the first day. The pathogenesis of leptospirosis in wild animals is unknown, and data from humans was adapted in order to define a reasonable period within

Table 2

Serogroups, numbers of positive animals and antibody titers detected by the microscopic agglutination test (MAT) performed in free-ranging wild species from the municipality of Botucatu, Brazil.

Species	Serogroup	N	Titers				
			100	200	400	800	1600
Coati ^a	Australis	5	1	3	1		
	Sejroe	4	1	1	1		1
	Icterohaemorrhagiae	3		2		1	
	Djasiman	2		1	1		
	Grippytyphosa	2		1	1		
	Autumnalis	1	1				
	Panama	1		1			
Crab-eating fox	Australis	2				2	
	Djasiman	1				1	
Crab-eating raccon	Icterohaemorrhagiae	1					1
Hoary fox	Djasiman	2	1	1			
Lesser grison ^a	Shermani	2	1	1			
	Djasiman	1	1				
	Australis	1		1			
	Canicola	1		1			
Maned wolf	Canicola	1				1	
	Djasiman	1	1				
Nine-banded armadillo	Djasiman	1	1				
Opossum ^a	Autumnalis	1	1				
	Canicola	1	1				
	Icterohaemorrhagiae	1	1				
	Grippytyphosa	1		1			
Southern tamandua	Sejroe	1					1
Tayra	Djasiman	1	1				
Total ^a	Djasiman	9	5	2	1	1	
	Australis	8	1	4	1	2	
	Icterohaemorrhagiae	5	1	2		1	1
	Sejroe	5	1	1	1		2
	Grippytyphosa	3		2	1		
	Canicola	3	1	1		1	
	Shermani	2	1	1			
	Autumnalis	2	2				
	Panama	1		1			

N: number of animals positive in MAT.

^a Includes animals that were positive for more than one serogroup, thus the sum of animals in this table is higher than those of Table 1.

which the tests used (serology and PCR, see below) would be capable of detecting positive animals after infection. Based on data published by Levett (2001), we established 10 days as a suitable period, which was subtracted from the sampling date.

In the laboratory, urine samples were washed to decrease DNA degradation during storage in a freezer. They were initially centrifuged at 12,851 G and the supernatant was discarded. The pellet was then washed with sterile phosphate-buffered saline pH 7.6 (PBS) using an automatic agitator (IKA[®]) for 10 s. The centrifugation and washing were repeated one more time, and the ensuing pellet was suspended in a final volume of 1 ml of sterile PBS to obtain a high concentration of sediments. Serum was obtained through centrifugation of blood. Urine and serum samples were then stored at -80°C until the time of analysis.

2.3. Serological diagnosis

The microscopic agglutination test (MAT) was used for making serological diagnoses. The serum samples were tested against 28 antigens of *Leptospira* spp., which represented 18 serogroups: Australis, Autumnalis, Ballum, Bataviae, Canicola, Celledoni, Djasiman, Grippytyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Pomona, Pyrogenes, Sejroe, Shermani and Andamana. Details of the

MAT procedure have previously been described (Fornazari et al., 2012). The results were expressed at serogroup level rather than at serovar level, due to cross-reactions that may occur among different serovars, as reported in the literature that we consulted (Levett, 2001; Haake and Levett, 2015). When an animal was positive for more than one serogroup, only the one with the highest titer was considered. However, if these serogroups presented the same titer, the animal was considered reactive to both of them.

2.4. Molecular diagnosis

A real-time PCR assay (PCR) was used to detect *Leptospira* DNA in urine samples. After thawing the samples, genomic DNA was extracted using a commercially available DNA extraction kit (Illustra[™] Blood Genomic Prep Mini Spin kit, GE Healthcare[®]) in accordance with the manufacturer's instructions. A local strain kept in EMJH medium, which had been isolated from a dog's urine (serovar Canicola), was used as the positive control, while sterile PBS was used as the negative control.

DNA amplification was performed using the StepOne[™] Plus Real Time PCR System equipment (Applied Biosystems[®]) and the StepOne 2.1 software. Primers targeting the *rrs* gene were used (Mérien et al., 1992). This gene is specific for the genus *Leptospira* and produces a 331-bp amplicon. Reactions were performed in disposable 96-well plates, free of DNase and RNase. The volume of each well was 20 μl , consisting of 2 μl of sample, 10 μl of Power SYBR[®] Green mix, 0.2 μl of each primer (10 $\mu\text{mol}/\mu\text{l}$) and 7.6 μl of Milli-Q water. The plates were sealed and subjected to the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min) and melting curve analysis ($60\text{--}95^{\circ}\text{C}$ for 55 min). All samples were processed in triplicates.

2.5. Genetic characterization

PCR amplicons were purified using the QIAquick[®] PCR Purification kit, in accordance with the manufacturer's instructions. These samples were sequenced in both directions by means of the Sanger sequencing method, using the same PCR primers and the Big Dye[®] Terminator Cycle Sequencing kit. The reactions were conducted in a thermocycler (Mastecycler[®] Eppendorf) and the products were analyzed in the 3500xL Genetic Analyzer (Applied Biosystems[®]). The quality of electropherograms was assessed using the Chromas Lite software (v 2.1.1). The Molecular Evolutionary Genetics Analysis (MEGA 7.0) was used to edit FASTA sequences and align them with other reference strains available in GenBank that had previously been described by Cerqueira et al. (2010). A phylogenetic tree was constructed using the neighbor-joining method, Kimura two-parameter model and 1000 bootstrap replications.

2.6. Statistical analysis

Prevalence was defined as the ratio of the number of positive animals to the number of sampled animals and its respective confidence intervals were estimated for each species using the binomial distribution. To evaluate the influence of different factors on the prevalence of *Leptospira*, two analysis methods were used. Initially, the results from the laboratory tests (MAT/PCR) were combined into a single value. A positive outcome was defined as positivity in at least one of the tests, and was modeled using exact logistic regression or penalized (Firth) logistic regression in the presence of total separation using R. Age (young vs. adult), sex (male vs. female), type of environment (urban vs. rural) and season (spring, summer, fall and winter) were considered to be explanatory factors in a species-specific model. A liberal *P*-value of 0.15 was initially considered significant. Significant predictors as well as all possible interactions were evaluated in the final models where statistical significance was considered at the 5% level. In the absence of

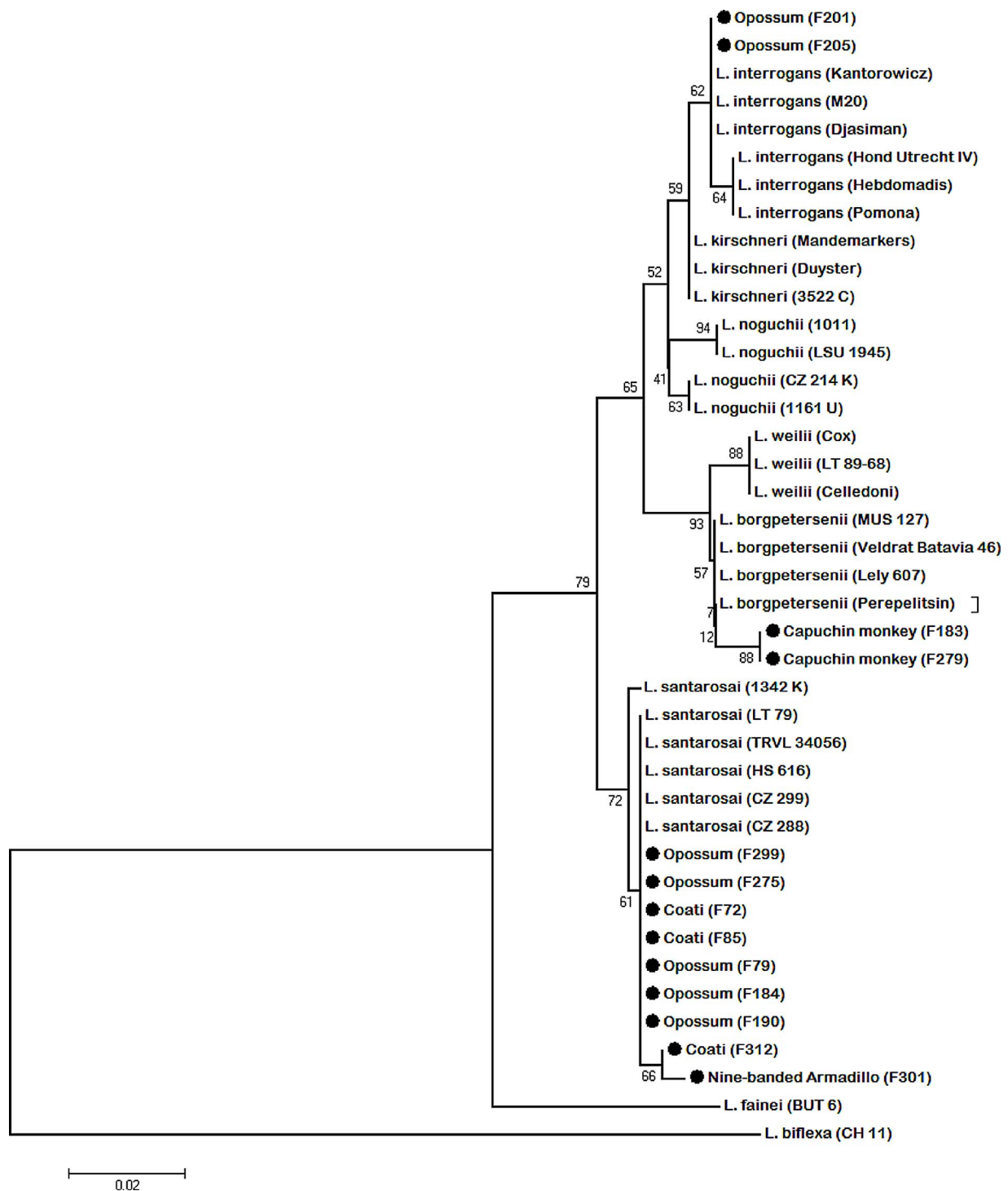


Fig. 1. Phylogenetic tree of the *rrs* gene sequence of *Leptospira*, including reference strains and sequences obtained from wild species in the municipality of Botucatu, Brazil (black circles). Strain name and individual identification of animals are given in parenthesis.

modification, simpler models were considered for assessment of confounding.

A second strategy was used for serogroups and genomospecies. Since a relatively low number of positive results was observed per outcome, Fisher's Exact test was used for each outcome and each explanatory factor, individually for different species. All statistical analyses were performed using the R software (R Development Core Team, 2008), considering a significance level of 0.05. *P*-values were adjusted for multiple comparisons using the method described by Benjamini and Hochberg (1995), through the *p.adjust* function in R. Odds ratios and

their respective 95% exact confidence intervals were estimated when statistical significance, defined as a *P*-value < 0.05, was present.

3. Results

The overall number of animals studied was 309, distributed among 16 species. The results regarding the number of individuals per species, MAT and PCR are detailed in Table 1. Opossums (*Didelphis albiventris*) and coatis (*Nasua nasua*) were the most numerous species sampled.

MAT showed that 33 animals were positive (11%), with titers

Table 3
Absolute and relative frequencies of *Leptospira* infection in coatis (*Nasua nasua*) and opossums (*Didelphis albiventris*) according to observed factors in the municipality of Botucatu, Brazil.

Species	Factor	Level	Result (%)		P	OR (95% CI)
			Negative	Positive		
Opossum	Sex	Male	88 (93.6)	6 (6.4)	0.66	–
		Female	97 (96.0)	4 (4.0)		
	Source	Rural	55 (91.7)	5 (8.3)	0.32	–
		Urban	130 (96.3)	5 (3.7)		
	Age	Young	121 (98.4)	2 (1.6)	0.01	7.48 (1.44–74.4)
		Adult	64 (88.9)	8 (11.1)		
	Season	Spring	49 (90.7)	5 (9.3)	0.22	–
		Summer	43 (97.7)	1 (2.3)		
		Fall	59 (93.7)	4 (6.3)		
		Winter	34 (100.0)	0 (0.0)		
Coati	Sex	Male	20 (66.7)	10 (33.3)	0.9	–
		Female	16 (61.5)	10 (38.5)		
	Source	Rural	41 (73.2)	15 (26.8)	^a	–
		Urban	0 (0.0)	0 (0.0)		
	Age	Young	16 (69.6)	7 (30.4)	0.69	–
		Adult	20 (60.6)	13 (39.4)		
	Season	Spring	16 (59.3)	11 (40.7)	0.8	–
		Summer	14 (66.7)	7 (33.3)		
		Fall	6 (75.0)	2 (25.0)		
		Winter	0 (0.0)	0 (0.0)		

OR: Odds Ratio.

^a Not calculated because animals were from just one type of environment.

Table 4
Absolute and relative frequency of infection by *Leptospira santarosai* genomospecies in opossums (*Didelphis albiventris*) according to observed factors in the municipality of Botucatu, Brazil.

Factor	Level	Result (%)		P	OR (95% CI)
		Negative	Positive		
Sex	Male	89 (94.7)	5 (5.3)	0.02	0.13 (0–0.99)
	Female	101 (100)	0 (0)		
Environment	Rural	57 (95.0)	3 (5.0)	0.17	–
	Urban	133 (98.5)	2 (1.5)		
Age	Young	122 (99.2)	1 (0.8)	0.06	–
	Adult	68 (94.4)	4 (5.6)		
Season	Spring	52 (96.3)	2 (3.7)	0.87	–
	Summer	43 (97.7)	1 (2.3)		
	Fall	61 (96.8)	2 (3.2)		
	Winter	34 (100)	0 (0)		

OR: Odds Ratio.

ranging from 100 to 1600. Serological reactions for nine serogroups were observed, with highest positivity for Djasiman and Australis (Table 2). Coati was the species with the highest prevalence of positive reactions (35.7%). Although high prevalence has also been observed in other species, these results were considered doubtful due to the small numbers of individuals sampled (see Table 1). This observation is also valid for PCR results.

PCR showed that 17 animals were positive (5.5%), with highest positivity for coatis (8.9%) and porcupines (15.3%). If MAT and PCR are considered to be complementary tests, the overall prevalence of positive animals was 16%. Only one animal (nine-banded armadillo) was positive for both tests.

Successful sequencing of PCR amplicons was obtained in relation to 13 animals, while the remaining four samples yielded electropherograms of poor quality, despite our efforts to improve the analysis (two porcupines and two coatis). Phylogenetic analysis revealed leptospires with high similarity to the genomospecies *L. santarosai* (n = 9), *L. interrogans* (n = 2) and *L. borgpetersenii* (n = 2) (Fig. 1).

The influence of selected variables in relation to opossums and coatis was tested. The remaining species were not tested because few individuals were sampled, which would significantly reduce the validity of the analysis. Higher prevalence of infection was observed among adult opossums than among young ones (Table 3). The analyses testing different serogroups and genomospecies indicated that the prevalence of *L. santarosai* in opossums was higher among males (Table 4). No other variables presented any significant differences ($P > 0.05$).

4. Discussion

The present study identified new species harboring *Leptospira* among wildlife in Brazil. Although no viable bacteria were investigated in our study, the presence of *Leptospira* DNA strongly suggests that these animals eliminate this pathogen in urine. This was the first study in Brazil conducted on a large population of wild species, using serological and molecular tests simultaneously. Interestingly, the overall prevalence of 16% matches a recent global assessment of *Leptospira* infection in vertebrates, which detected 15% in the majority of mammalian families (Andersen-Ranberg et al., 2016).

The prevalence of infection varied greatly among the species. Although leptospirosis is a disease linked to environmental conditions, our results suggest that there is a difference in prevalence at taxonomic level. We considered this hypothesis because the sites at which the animals were caught were very similar regarding the environmental characteristics (except in the case of opossums from the urban zone), and also because leptospires may have a specific evolutionary relationship with their carrier hosts (Murray et al., 2015). Such host-pathogen specificity could predispose towards chronic infection, and thus influence the MAT and PCR results. However, the variation in prevalence between species may be influenced by environmental factors, although we were unable to identify them. If this was the case, these variables were present on a very small scale, or were even unknown. Animal ecology is another important issue to be considered, and it is possible that the risk of infection in a particular area will depend on features such as home range, vertical use of habitat, foraging behavior, attraction towards aquatic habitats, movement patterns and contact with other animals (this was the reason why we considered it appropriate to run statistical models separately for each species). Nevertheless, we were unable to determine the origin of such great differences in prevalence between different species, and it is possible that all the abovementioned issues may interact simultaneously.

Infection by several serogroups was confirmed, thus implying that multiple epidemiological cycles exist in the region studied here. Djasiman and Australis were the most common serogroups, which indicates that there is a high level of exposure among these wild species. These two serogroups were recently detected in wild animals in both France and Brazil (Ayril et al., 2016; Vieira et al., 2016). Although only rarely reported, Djasiman was previously associated with human leptospirosis (Ismail et al., 2006; Héry et al., 2015). In Brazil, little is known about which leptospires most affect humans. Accurate information is available from the municipality of Salvador, where the *L. interrogans* serogroup Icterohaemorrhagiae is the most important agent causing leptospirosis among slum residents (Hagan et al., 2016). Icterohaemorrhagiae was the third most frequent serogroup in the present study, which indicates that these animals are possibly involved in the life cycle of pathogenic leptospires. However, it is unknown whether the serogroups detected through MAT might be carried by these animals and shed into the environment, since no phenotypic characterization of bacteria from urine was performed. The animals studied here may be poor reservoirs, unable to develop chronic infection, or they may shed leptospires, but at low bacterial concentrations and only for very short periods. Along this line of thinking, it is also possible that the *L. interrogans* serogroup Icterohaemorrhagiae is indeed the most important pathogen causing human leptospirosis in Brazil, despite the

lack of national studies that include genotypic and phenotypic characterization of bacteria. In this case, other serogroups would be less important for public health, including Djasiman and Australis, which were detected at high frequencies in the present study. Therefore, the status of wild animals as reservoirs of leptospires needs to be taken into consideration, but their relevance in relation to public health issues is still a matter of some doubt. Comparisons between isolates from wild animals and humans would help to answer this question.

Results from opossums and coatis, which were sampled in large numbers, deserve special attention. We considered that the prevalence of *Leptospira* in opossums was surprisingly low. Opossums are very opportunistic and are commonly found in rural and urban areas all over Brazil (Fornazari et al., 2011). They often share the same ecological niche that rodents occupy, searching for domestic garbage and nesting places. Therefore, environmental infection with leptospires was expected to be high, especially in relation to the Icterohaemorrhagiae serogroup. However, the results were contrary to this theory, and we can conclude that opossums have a low rate of infection in the region studied, despite occurrences of leptospirosis in Botucatu (Langoni et al., 2008; Coiro et al., 2012; Fornazari et al., 2012; Kikuti et al., 2012) and the presence of large populations of synanthropic rodents (personal communication from public health authorities). Because *Leptospira* DNA was found in opossum urine, this species may indeed act as a renal carrier of leptospirosis. In southern Brazil, *Leptospira* was successfully isolated from the urine of an opossum (*D. albiventris*), and its pathogenicity was demonstrated through inoculation in hamsters (Jorge et al., 2012). However, the low prevalence that we detected through PCR suggests that opossums have a limited role in environmental contamination. There is a common belief in Brazil that opossums are dangerous carriers of zoonoses, especially in urban areas. Regarding leptospirosis, our results contradict these thoughts. Likewise, in preliminary studies, we found low prevalence of toxoplasmosis (Fornazari et al., 2011), leishmaniasis (Paiz et al., 2015) and brucellosis (Antunes et al., 2010). Thus, contrary to our initial thoughts, so far there is no evidence that opossums are important carriers of zoonoses in Botucatu. One last note on this issue concerns the great phylogenetic distance between opossums (infraclass Marsupialia) and the remaining mammals that were sampled (infraclass Placentalia). Such an ancient evolutionary divergence could imply distinct physiological and ecological features that influence infection outcomes.

Coatis are also a very common species in Brazil and often develop synanthropic behavior. They can be found living in large groups, foraging near human habitations and parks, especially at the limits between urban and rural areas. The prevalence of *Leptospira* in this species was high, thus revealing that these animals had contact with many serogroups, and a significant proportion of the individuals caught were found to be shedding leptospires (8.9% in PCR). As discussed above in relation to opossums, further investigations are needed in order to confirm the relevance of coatis for public health. Nevertheless, we consider that two ecological features possibly involved in the high prevalence of *Leptospira* among coatis are noteworthy: their large home range and their intense foraging behavior on the ground. Coatis use large territories (Beisiegel and Mantovani, 2006), which can increase the likelihood of contact with areas contaminated with leptospires. During our field work, we indeed confirmed that animal groups occupied extensive territories, moving through farms, periurban areas and forests. Thus, it seems very likely that the risk of infection could be enhanced through contact with such a great diversity of environments, where many water bodies and domestic and wild animals can be found. Moreover, during foraging, coatis have intense contact with soil, in their search for insects, small vertebrates and fruits. This behavior could predispose towards infection because of the high degree of contact between their oral mucosae and the ground surface. Other studies have also pointed out that wild species that have intense contact with soil, like rodents and armadillos, have high exposure to certain pathogens (Bagagli et al., 1998; Drancourt et al., 2006).

Results relating to small-sized samples should always be carefully interpreted, since they cannot properly represent a population. Nonetheless, our results relating to some species were very interesting, such as those from lesser grisons, capuchin monkeys, crab-eating foxes and hoary foxes. All of these species were sampled in small numbers but presented very high prevalence of *Leptospira*, especially through MAT, thus indicating high levels of exposure to leptospires. These animals were trapped in more than one place, on different dates, which decreases the possible biases relating to geographic location and seasonality, respectively (the only exception was in relation to two capuchin monkeys that were trapped at the same site and probably belonged to the same group). Such high prevalence was surprising, but the assumption that these species have high levels of infection is doubtful because only a few individuals were investigated. On the other hand, if these species are pooled with coatis, they form a group that is included in the order Carnivora (except for capuchin monkeys). Very high prevalences of *Leptospira* in wild carnivores in Brazil have previously been reported, using MAT (Jorge et al., 2011; Vieira et al., 2016). It is possible that this taxonomic group is more exposed to leptospires than others, since they prey on other animals and hence could become infected through direct contact with the secretions and tissues of other animals, including rodents. As discussed in relation to coatis, we consider that the large home range used by carnivores is an important factor, which could enhance the likelihood of infection.

Genetic characterization has revealed the diversity of *Leptospira* genomospecies circulating among wild animals, with predominance of bacteria close to *L. santarosai*. Studies using molecular tools are scarce in Brazil, thus hindering deeper discussion on this topic. Nevertheless, occurrences of *L. santarosai* in different animal species in Brazil, like goats (Lilenbaum et al., 2015), dogs (Miotto et al., 2016) and cattle (Hamond et al., 2015), have been confirmed in the literature, which indicates that this genomospecies is common in this country. Detection of leptospires genetically close to *L. borgpetersenii*, which has only been reported in Brazil a few times, is also noteworthy. Here we described this bacterium infecting two capuchin monkeys.

A secondary goal of this study was to test the influence of different factors on the prevalence of *Leptospira* infection. Adult opossums presented higher prevalence than young ones (OR = 7.48). The effect of age in relation to the epidemiology of leptospirosis has been poorly assessed, particularly among wild species. Previous studies found similar results from Norway rats and, since infection is chronic in this species, this finding is usually attributed to these rodent's higher likelihood of encountering the pathogen during their life span (Easterbrook et al., 2007; Krjogaard et al., 2009; Himsforth et al., 2013). A similar outcome may occur among opossums. Moreover, because rodents and marsupials are taxonomically distant, it needs to be considered that age may influence *Leptospira* infection in different species of mammals. There was also a statistically significant difference in infection by *L. santarosai* between male and female opossums, which was higher in males. As previously confirmed, there are sex-related differences in infection by several types of pathogens (Klein, 2000). Males of many species are known to be more susceptible because of the immunosuppressive effects of androgen hormones. Behavior among males also includes higher aggression levels and larger home ranges, thereby increasing the risk of exposure to pathogens. This information can therefore assist professionals working with leptospirosis control, or wildlife management, since adult male individuals can be considered to be a priority when leptospirosis reservoirs are being investigated.

There was no statistical significant difference in the prevalence of infection among opossums, between rural and urban areas. It is possible that, in the study region, environmental conditions do not play a major role in infection among opossums. Another explanation is that the small sample size of individuals from rural areas ($n = 60$) was insufficient to provide reliable results. We tend to believe the second hypothesis more, since leptospirosis is a disease that is strongly linked to environmental factors, and the two areas investigated here displayed large differences

in their characteristics.

There was also no association regarding season. *Leptospira* infection is usually higher in rainy periods, which in Brazil occur during summer. Similar results were obtained by [Cosson et al. \(2014\)](#), from studying wild rodents in southeast Asia, in which *L. borgpetersenii* infection was common in both wet and dry habitats. A recent meta-analysis approach also did not find any positive association between climatic variables and infection among carnivores ([Andersen-Ranberg et al., 2016](#)). The epidemiology of leptospirosis is complex and probably varies greatly among different types of environment, animal species and bacterial strains. Thus, climate and humidity may not always be determining factors.

Finally, we discuss some aspects of the laboratory tests that were used. Although MAT is considered to be the reference serological test, it has several limitations relating to false negative results: animals can become infected by serogroups that are not included in the MAT collection; sometimes, a given serogroup cannot be represented by a single antigen that is included in MAT, because cross-reactions between strains of the same serogroup may be weak ([Levett, 2001](#)); infected animals may have antibody titers below the widely accepted minimum titer of 100 ([Ellis, 2015](#)); strains that are highly adapted to the animal reservoir may not produce any immunological response ([Himsworth et al., 2013](#)); and there is evidence of attenuation of antigens after several years of successive in vitro passages ([Tulsiani et al., 2011](#)). Thus, MAT frequently underestimates the prevalence. For instance, seroprevalence in opossums may be higher when a local strain isolated from this species is used as the antigen ([Jorge et al., 2012](#)). In addition to the issues relating to diagnostic sensitivity, MAT does not allow detection of whether a serological reaction that is positive for more than one serovar is due to cross-reactions between antigens or whether it is due to co-infections by distinct leptospires. In this study, we only considered the serogroup with the highest titer. However, the possibility of co-infections should not be ruled out, given the cosmopolitan nature of this pathogen in tropical regions. Regarding PCR, use of kidney samples would probably increase the sensitivity of the diagnosis, because shedding of leptospires in urine occurs intermittently. Also, in almost all the animals examined, the use of furosemide was indispensable, since they had little or no urine in the bladder. Thus, elimination of bacteria probably did not occur naturally and lower-than-normal concentration ensued. This was evident from the transparency and lack of color in the urine samples. The kidneys were not used as samples because it would have been necessary to sacrifice the animals, which was undesirable. Thus, in this light, the true prevalence of infected animals is probably higher than what we detected.

The poor agreement between MAT and PCR was not surprising, and this finding in both domestic and wild species is corroborated in the literature ([Langoni et al., 2008](#); [Fornazari et al., 2012](#); [Vieira et al., 2016](#)). Several factors can explain these results. Infected animals can produce antibodies and show positive results through MAT, but chronic infection and positivity in PCR will depend mostly on the specificity between animals and bacterial strains ([Murray et al., 2015](#)). In the absence of such specificity, kidney and urine samples may be negative through PCR. The opposite outcome is also common, in which an animal is PCR-positive and MAT-negative. Carriers of leptospirosis may have antibody titers below the detection threshold (100), or the antigens used in MAT may not be representative of the infecting leptospires. Several factors may explain the poor agreement between MAT and PCR, including the technical limitations that were already discussed. Therefore, simultaneously use of both laboratory tests may be very useful for characterizing the infection status in a given population.

5. Conclusions

Wild species were identified as reservoirs of *Leptospira*, thus providing new information on the epidemiology of this pathogen. Great differences in prevalence of infection were observed between species

and, from this, coatis and other carnivores are indicated as priorities for further investigations. Despite the synanthropic nature of opossums, it is unlikely that they play a role as reservoirs of leptospirosis in the region studied here. Most of the leptospires were included in *L. santarosai* genomospecies and Djasiman/Australis serogroups. Age and sex were important factors influencing infection in opossums. These data can assist in leptospirosis surveillance, characterize the health status of wildlife and point towards new directions for future studies.

Conflict of interest

We declare that there is no conflict of interest.

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